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Virology 318 (2004) 556–565

VIROLOGY

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Interaction between C/EBPβ and Tax down-regulates human T-cell leukemia virus type I transcription

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Received 5 June 2003; returned to author for revision 9 October 2003; accepted 20 October 2003

Abstract

The human T-cell leukemia virus type I (HTLV-I) Tax protein *trans*-activates viral transcription through three imperfect tandem repeats of a 21-bp sequence called Tax-responsive element (TxRE). Tax regulates transcription via direct interaction with some members of the activating transcription factor/CRE-binding protein (ATF/CREB) family including CREM, CREB, and CREB-2. By interacting with their ZIP domain, Tax stimulates the binding of these cellular factors to the CRE-like sequence present in the TxREs. Recent observations have shown that CCAAT/enhancer binding protein β (C/EBPβ) forms stable complexes on the CRE site in the presence of CREB-2. Given that C/EBPβ has also been found to interact with Tax, we analyzed the effects of C/EBPβ on viral Tax-dependent transcription. We show here that C/EBPβ represses viral transcription and that Tax is no more able to form a stable complex with CREB-2 on the TxRE site in the presence of C/EBPβ. We also analyzed the physical interactions between Tax and C/EBPβ and found that the central region of C/EBPβ, excluding its ZIP domain, is required for direct interaction with Tax. It is the first time that Tax is described to interact with a basic leucine-zipper (bZIP) factor without recognizing its ZIP domain. Although unexpected, this result explains why C/EBPβ would be unable to form a stable complex with Tax on the TxRE site and could then down-regulate viral transcription. Lastly, we found that C/EBPβ was able to inhibit Tax expression in vivo from an infectious HTLV-I molecular clone. In conclusion, we propose that during cell activation events, which stimulate the Tax synthesis, C/EBPβ may down-regulate the level of HTLV-I expression to escape the cytotoxic-T-lymphocyte response.

Keywords: HTLV-I; Tax; T cells

Introduction

Human T-cell leukemia virus type I (HTLV-I) causes adult T-cell leukemia and tropical spastic paraparesis. The viral Tax protein plays a critical role in the proliferation and the transformation of HTLV-I-infected T cells (Basbous et al., 2002). Tax interferes with cell growth control pathways through activation of NF-κB (Xiao et al., 2001), E2F (Lemasson et al., 1998), and AP-1 (Iwai et al., 2001), and through inactivation of p53 (Suzuki et al., 1999). This 40-kDa protein is also involved in the *trans*-activation of proviral transcription (Thébault et al., 2001). Tax stimulates viral transcription via protein–protein interactions with different members of the activating transcription factor/CRE-binding protein (ATF/CREB) family including CREM (Suzuki et al., 1993), CREB (Franklin et al., 1993; Yin and Gaynor, 1996; Zhao and Giam, 1992), and CREB-2 (Gachon et al., 1998; Lemasson et al., 2002; Reddy et al., 1997). These cellular factors, which are characterized by basic leucine-zipper (bZIP) C-terminal structures required for DNA binding and protein dimerization, bind to the Tax-responsive elements (TxREs) (Franklin et al., 1993; Gachon et al., 2000; Zhao and Giam, 1992), located in the U3 region of the long terminal repeat (LTR) promoter. The TxREs are three 21-bp repeats composed of a central cAMP-response element (CRE)-like motif, flanked by a short run of guanine and cytosine base pairs. Tax stimulates viral transcription first by promoting the homodimerization of the ATF/CREB factors (Gaudray et al., 2002; Wagner and Green, 1993) and then by stabilizing the LTR-bound complexes through direct

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0042-6822/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2003.10.027

contacts with nucleotides flanking the CRE-like motifs (Kimzey and Dynan, 1998; Lenzmeier et al., 1998; Lundblad et al., 1998). The ZIP structure is absolutely required both for interactions with Tax and for increased binding of the ATF/CREB factors to the viral promoter (Gachon et al., 2000; Yin et al., 1995). Afterwards, the TxRE-associated Tax molecule recruits the transcriptional coactivator CREB-binding protein (CBP) (Kwok et al., 1996) responsible for the core histone tail acetylation (Gachon et al., 2002; Georges et al., 2002).

The ATF/CREB factors are not the only bZIP factors capable of binding to the CRE motif of the HTLV-I LTR. c-Jun, that belongs to the activator protein-1 (AP-1) family, activates viral transcription through the TxRE sequences (Jeang et al., 1991; Lemasson et al., 2002). However, no direct interaction between c-Jun and Tax has been found, suggesting that AP-1 is obviously involved in basal transcription of the HTLV-I genome. Another well-characterized example of bZIP factors binding to the CRE site is the CCAAT/enhancer binding protein (C/EBP) family (Bakker and Parker, 1991). The latter includes at least six different proteins (Lekstrom-Himes and Xanthopoulos, 1998), many of which are important activators of transcription. All members share a highly homologous C terminus that contains the bZIP domain involved in their homo- or heterodimerization. Among the C/EBP protein family members, C/EBPβ, also called NF-IL6, LAP, or CRP2, can form a heterodimer with CREB-2. This heterodimer does not bind to the CCAAT box, but it binds to the CRE site with high affinity (Podust et al., 2001). In addition, C/EBPβ has also been shown to physically interact with Tax to activate transcription from the promoter of human prointerleukin-1β gene (Tsukada et al., 1997).

In light of these results, we analyzed the effects of C/EBPβ on the regulation of HTLV-I transcription. Unexpectedly, we observe that C/EBPβ down-regulates HTLV-I transcription in the presence of Tax. We demonstrate that Tax is no longer able to *trans*-activate viral transcription because the presence of C/EBPβ blocks the recruitment of Tax on the TxREs. To understand this negative effect, we analyzed the interactions between Tax and C/EBPβ by glutathione *S*-transferase (GST)-pull down assay. By this approach, we show that Tax interacts with C/EBPβ but that the ZIP domain of C/EBPβ is not involved in this interaction as it has been previously demonstrated for other cellular bZIP factors (Gachon et al., 2000; Yin et al., 1995). By cotransfection assays in CEM cells, we confirm that the bZIP domain of C/EBPβ is unable to down-regulate Tax-dependent transcription. On the other hand, the truncated mutant C/EBPβ-ΔZIP remains capable of repressing viral transcription. At last, we demonstrate that Tax, reciprocally, can antagonize the transcriptional activity of C/EBPβ on CAAT/enhancer sites. In conclusion, we propose that C/EBPβ, by preventing the binding of Tax to the TxREs, behaves as a transcriptional repressor which could control HTLV-I expression in vivo.

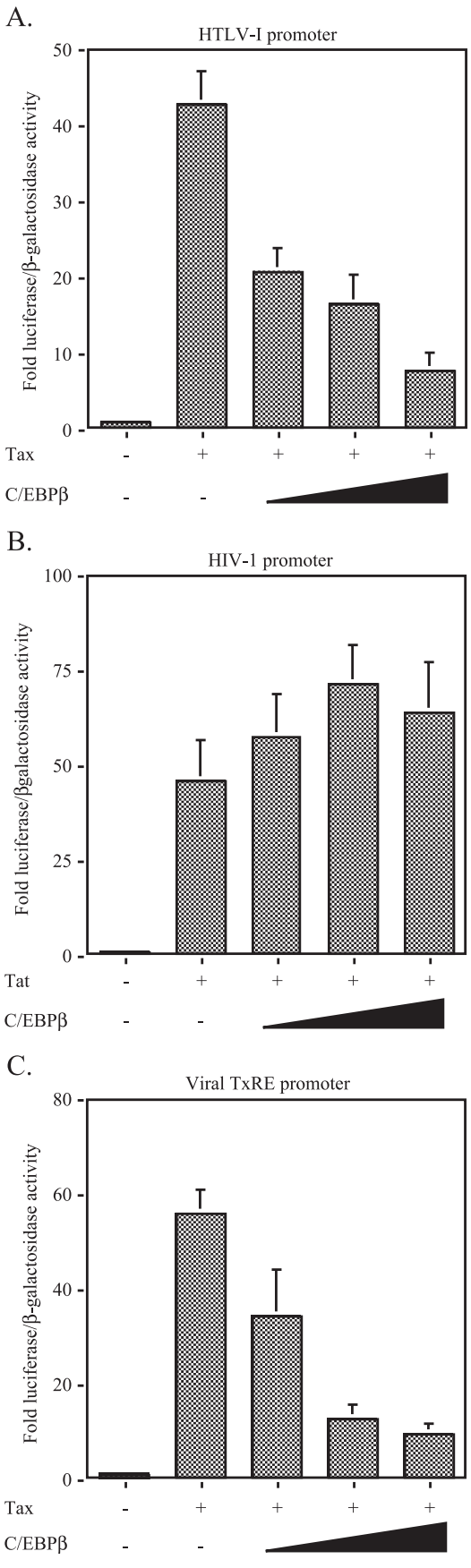
Results

C/EBPβ down-regulates HTLV-I transcription

By screening a cDNA library constructed from the HTLV-I-infected MT2 cell line (Gachon et al., 1998) with the bZIP domain of CREB-2 as a bait, we isolated cDNA clones (data not shown) coding for five different bZIP factors including HTLV-I bZIP factor (HBZ) (Gaudray et al., 2002), NF-E2 related factor (Nfr2) (He et al., 2001), C/EBPβ (Podust et al., 2001), C/EBPγ (Vinson et al., 1993), and C/EBP-homologous protein (CHOP) (Gachon et al., 2001). Among them, we were particularly interested by C/EBPβ. Indeed, this bZIP factor has been described to interact not only with CREB-2 (Podust et al., 2001; Vinson et al., 1993) but also with Tax (Tsukada et al., 1997). Moreover, the C/EBPβ-CREB-2 heterodimer has been described to form a stable complex on the CRE site (Podust et al., 2001). In light of these observations, we analyzed the C/EBPβ effect on HTLV-I transcription in a cotransfection assay. CEM cells were transfected with a luciferase reporter construct carrying the HTLV-I promoter and increasing amounts of C/EBPβ in the presence of a Tax expression vector pSG-Tax. As shown in Fig. 1A, Tax alone activated the luciferase reporter gene expression by 43-fold but this stimulation was inhibited in the presence of C/EBPβ (only an 8-fold stimulation). To be sure that this inhibition was not due to a nonspecific effect of C/EBPβ on cellular transcription, C/EBPβ was also tested in CEM cells with the human immunodeficiency virus type 1 (HIV-1) promoter in the presence of the HIV-1 activator Tat. Under these conditions, C/EBPβ did not down-regulate Tat-stimulated HIV-1 transcription (Fig. 1B), confirming that the decrease in the *trans*-activation by C/EBPβ from the HTLV-I promoter in the presence of Tax was specific. Thus, C/EBPβ is able to down-regulate HTLV-I transcription.

C/EBPβ blocks the recruitment of Tax on TxRE

We have already described the repression of HTLV-I transcription by cellular factors able to heterodimerize with CREB-2, for example, CHOP and HBZ (Gachon et al., 2001; Gaudray et al., 2002). These factors down-regulate viral transcription by forming heterodimers with CREB-2 that are no longer capable of binding to the CRE site present in the TxREs. It is unlikely that C/EBPβ inhibits viral transcription by the same mechanism because the C/EBPβ-CREB-2 heterodimer has been demonstrated to bind to the CRE motif (Podust et al., 2001). To be sure, however, that other regulatory sequences present in the HTLV-I LTR could not be responsible for the repression, the C/EBPβ effect was tested in CEM cells on a luciferase reporter construct carrying a synthetic promoter containing three tandem copies of the promoter-proximal TxRE, also called TxRE III. Fig. 1C shows that this reporter was stimulated 56-fold with pSG-Tax alone but only 9-fold in the presence



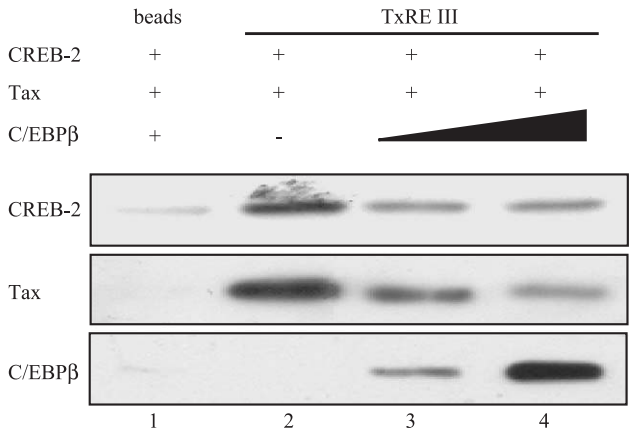


Fig. 2. C/EBPβ abolishes the binding of Tax to TxRE III. A biotinylated oligonucleotide (100 ng) corresponding to TxRE III was incubated with 6xHis-tagged CREB-2 (50 ng) and Tax (50 ng) in the absence (lane 2) or the presence (lanes 3 and 4) of purified bacterially GST-C/EBPβ (50 ng and 100 ng). The complexes were collected on streptavidin beads, and the proteins bound to the beads were analyzed by Western blotting with either anti-CREB-2 (top panel), anti-Tax (middle panel), or anti-C/EBPβ (bottom panel). Lane 1 corresponds to incubation of CREB-2, Tax, and C/EBPβ with streptavidin beads.

of C/EBPβ. This result confirms the involvement of the TxREs motifs in the repression of HTLV-I transcription by C/EBPβ.

To further investigate how C/EBPβ is able to inhibit HTLV-I transcription, we used the streptavidin–biotin complex assay that we have already used for the characterization of the complexes formed among Tax, CREB-2, and TxRE III (Gachon et al., 2000; Gaudray et al., 2002). A double-stranded oligonucleotide corresponding to the HTLV-I TxRE III site was incubated with Tax and CREB-2 in the absence or the presence of C/EBPβ. As shown in Fig. 2 (compare lanes 2 and 3), Tax and CREB-2 binding to TxRE III decreases in the presence of C/EBPβ. However, whereas Tax binding decrease is proportional to the quantity of C/EBPβ, the addition of higher concentration of C/EBPβ does not modify the quantity of CREB-2 bound to TxRE III (compare lanes 3 and 4 of Fig. 2). This result can be

Fig. 1. C/EBPβ down-regulates Tax-dependent transcription. CEM cells were cotransfected with the following: (A) 2 μg of HTLV-I LTR-luciferase, 5 μg of pACβ1 (β-galactosidase-containing reference plasmid), 1 μg of Tax-expression vector pSG-Tax, and 0, 1, 5, or 10 μg of pcDNA-C/EBPβ; the luciferase values are expressed as fold increase relative to that of cells transfected with pSG, pcDNA, and HTLV-I LTR-luciferase; (B) 2 μg of HIV-1 LTR-luciferase, 5 μg of pACβ1, 1 μg of the Tat-expression vector pBg312HIV-1Lai-Tat, and 0, 1, 5, or 10 μg of pcDNA-C/EBPβ; the luciferase values are expressed as fold increase relative to that of cells transfected with HIV-1 LTR-luciferase without Tat and C/EBPβ; (C) 2 μg of HTLV-I TxRE-luciferase, 1 μg of Tax expression vector pSG-Tax, and 0, 1, 5, or 10 μg of pcDNA-C/EBPβ; the luciferase values are expressed as fold increase relative to that of cells transfected with pSG-5, pcDNA, and HTLV-I TxRE-luciferase. For all the cotransfections, the total amount of DNA in each series of transfection was equal, the balance being made with the empty plasmids and the luciferase values were normalized for β-galactosidase activity. Values represent the mean ± SD (n = 3).

explained by the fact that the C/EBPβ-CREB-2 heterodimer forms a more stable complex on the CRE site than the CREB-2 homodimer does (Podust et al., 2001). Moreover, we also detected C/EBPβ bound to TxRE III (Fig. 2, lanes 3 and 4); indeed, the C/EBPβ homodimer is capable of binding to both CRE and CCAAT sequences with comparable affinity (Podust et al., 2001). In conclusion, our results show that C/EBPβ inhibits Tax from binding to TxRE III.

Tax does not interact with the bZIP domain of C/EBPβ

To better understand why C/EBPβ inhibits Tax, we analyzed the interactions between C/EBPβ and Tax by using the GST-pull down assay. The two C/EBPβ cDNAs isolated by the two-hybrid approach, C/EBPβ-ΔAD and C/EBPβ-bZIP (Fig. 3A), were subcloned into the pGEX plasmid that expresses the *Schistosoma japonicum* GST.

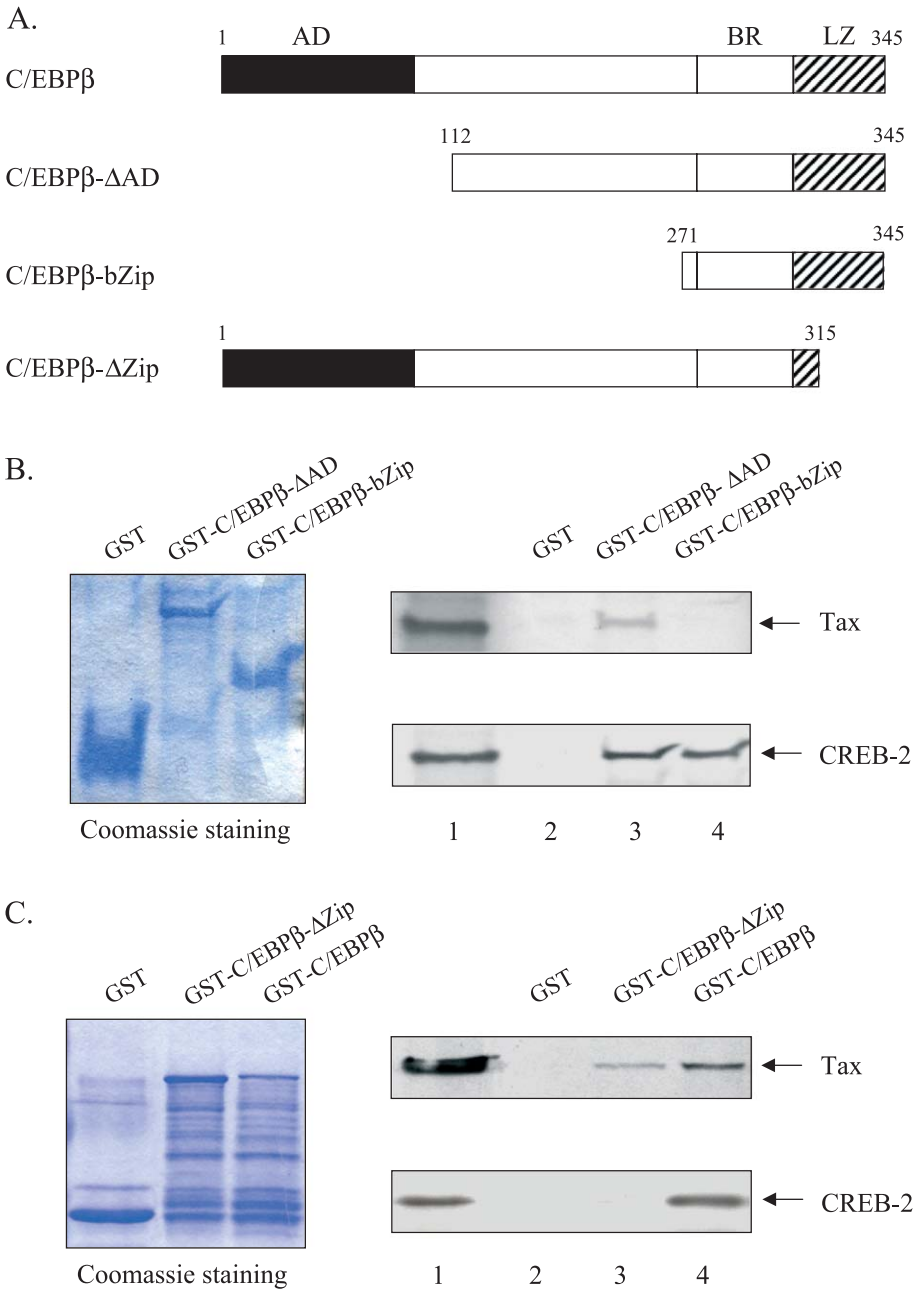


Fig. 3. The central region of C/EBPβ is involved in the interaction with Tax. (A) Schematic of C/EBPβ proteins fused to the GST, including C/EBPβ-ΔAD and -bZIP isolated by the two-hybrid approach by using the bZIP domain of CREB-2 as a bait, and the C-terminal truncated mutant C/EBPβ-ΔZIP are indicated. (B and C) Tax binds to the central domain of C/EBPβ. [^{35}S]-labeled Tax (top panel, lane 1) or CREB-2 (bottom panel, lane 1) was incubated with GST alone (lane 2) or the indicated GST-C/EBPβ proteins (lanes 3 and 4). The bound proteins were analyzed by 10% SDS-PAGE followed by autoradiography. Coomassie-stained SDS-PAGE showing the GST fusion proteins are also shown.

Equal amounts of the C/EBPβ proteins fused to GST were immobilized on glutathione Sepharose beads and incubated with Tax translated in the presence of [^{35}S]methionine and [^{35}S]cysteine. The N-terminal truncation of C/EBPβ (C/EBPβ-ΔAD, Fig. 3B, lane 3), with a deletion of 111 residues, did not abolish the interaction between Tax and C/EBPβ. On the other hand, the mutant with a further deletion extended into residue 270, but still possessing the ZIP domain, was unable to interact with Tax (C/EBPβ-bZIP, Fig. 3B, lane 4), demonstrating that the central region of C/EBPβ was responsible for the interaction with Tax. This result was unexpected because it had been found until now that Tax bound to the ZIP domain of the Tax-interacting bZIP factors (Gachon et al., 2000; Yin et al., 1995). Therefore, we first performed the same experiment with [^{35}S]-labeled CREB-2 to be sure that the ZIP domain of our GST-C/EBPβ-bZIP fusion protein was functional. As expected, CREB-2 strongly interacted with C/EBPβ-bZIP (Fig. 3B, lane 4). Then, we produced another C/EBPβ mutant, C/EBPβ-ΔZIP, with a deletion of 30 residues from the C terminus (Fig. 3A). This mutant was unable to interact with CREB-2 (Fig. 3C, lane 3), confirming that the ZIP structure was destroyed. On the other hand, this mutant remains able to interact with Tax (Fig. 3C). Taken together, our results confirm that Tax is able to interact with C/EBPβ as already demonstrated by Tsukada et al. (1997), but that the ZIP domain is not required for the direct interaction between Tax and C/EBPβ.

To confirm these results in vivo, CEM cells were cotransfected with the two mutants C/EBPβ-ΔZIP and -bZIP in the presence of Tax and a luciferase reporter construct carrying TxRE III motifs in a cotransfection assay. As shown in Fig. 4A, C/EBPβ-ΔZIP was able to inhibit Tax-dependent transcription as C/EBPβ does. On the other hand, no repression was detected with C/EBPβ-bZIP, although it was stably expressed in transfected cells (Fig. 4B). Taken together, our results show the bZIP domain of C/EBPβ is not involved in the repression of Tax activity. Moreover, we demonstrate that the negative effect of C/EBPβ was not due to an interaction with another endogenous bZIP factor such as CREB-2. Moreover, our data confirm the results obtained by the in vitro approaches because only the mutant able to interact with Tax, C/EBPβ-ΔZIP, inhibits TxRE-dependent transcription.

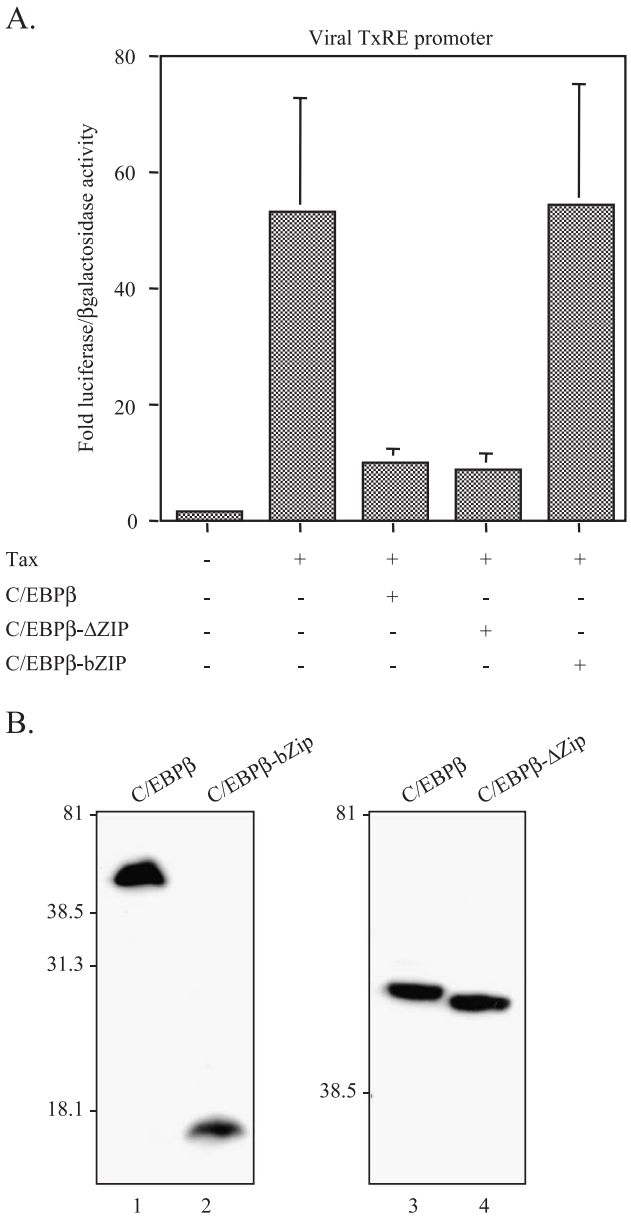
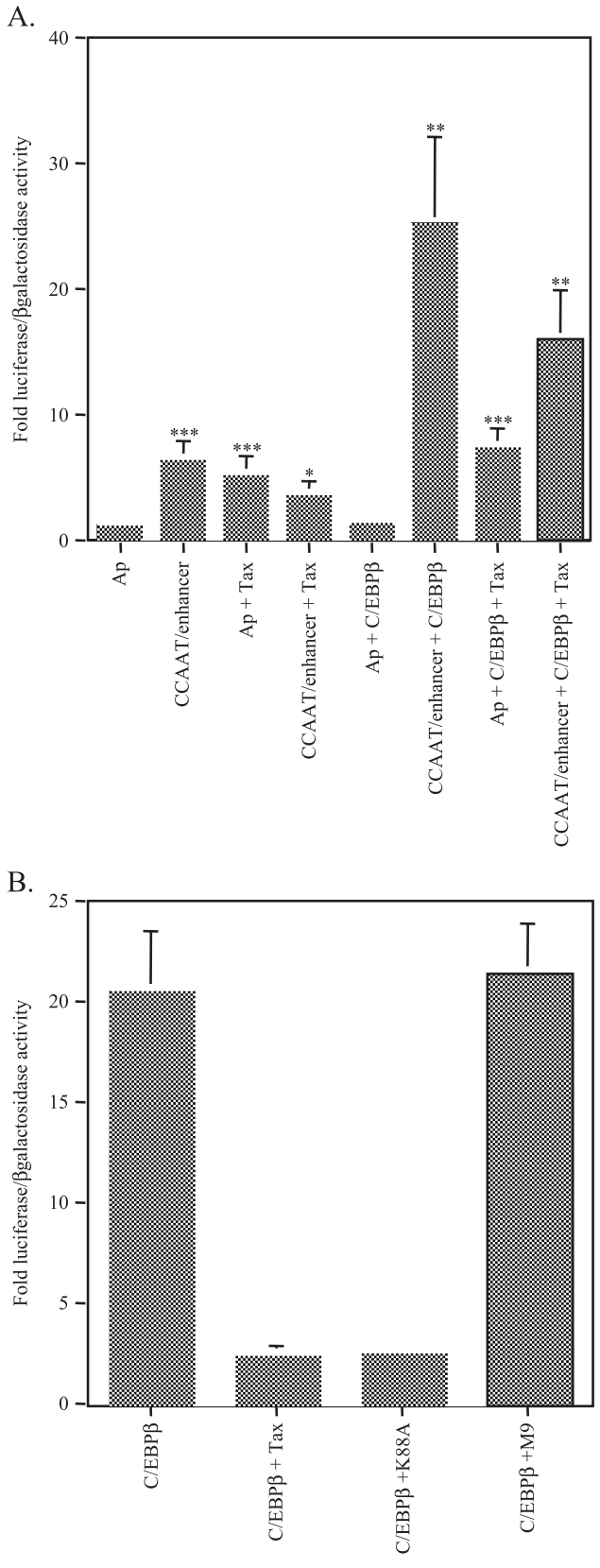


Fig. 4. The bZIP domain is not involved in the repression of Tax-dependent transcription by C/EBPβ. (A) CEM cells were cotransfected with 2 μg of HTLV-I TxRE-luciferase, 1 μg of Tax expression vector pSG-Tax, and 3 μg of pcDNA3.1/His expressing either C/EBPβ, or C/EBPβ-ΔZIP, or and C/EBPβ-bZIP. Values represent the mean ± SD (n = 3). (B) The C/EBP mutant proteins are stably expressed in vivo. Expression of the wild type (lanes 1 and 3) and truncated (lanes 2 and 4) C/EBPβ proteins were analyzed by 18% SDS-PAGE for transfections with C/EBPβ-bZIP (lanes 1 and 2) and 10% SDS-PAGE for transfections with C/EBPβ-ΔZIP (lanes 3 and 4), followed by Western blotting by using the anti-Xpress serum. The positions of protein molecular size standards are indicated (in kilodaltons).

The transcription function of C/EBPβ is reduced in the presence of Tax

As C/EBPβ was able to inhibit Tax activity, it was interesting to know whether the transcriptional repression could be reciprocal. The transcriptional activity of C/EBPβ in the presence of Tax was measured by using pE$_4$APLuc, a C/EBP-responsive luciferase reporter construct, which contains four tandem C/EBP sites cloned upstream of a minimal alkaline phosphatase promoter driving luciferase gene expression, and the control vector pAPLuc containing a minimal alkaline phosphatase promoter (Cooper et al., 1995). Fig. 5A clearly shows that pE$_4$APLuc was active in CEM cells (about 6-fold). On the other hand, the results obtained after cotransfection with pSG-Tax were more complex. Indeed, in the presence of Tax and pE$_4$APLuc, the luciferase activity was stimulated 3.5-fold but this stimulation was not due to the C/EBP sites because Tax was able to stimulate the

minimal alkaline phosphatase promoter (about 5-fold). Taken together, these data suggest that Tax down-regulates the activity of endogenous C/EBP factors.



To study the possible involvement of C/EBPβ in the repression by Tax, C/EBPβ and pE_4APLuc were cotransfected in the absence or presence of Tax. The reporter plasmid was stimulated 25-fold with C/EBPβ alone but only 16-fold in the presence of C/EBPβ and Tax (Fig. 5A). This repression of C/EBPβ activity could seem to be weak, but if we consider the Tax-stimulation of the alkaline phosphatase promoter, this inhibition is clearly significant. Such a repression of the transcriptional function of cellular factors by Tax has been described several times and it has been demonstrated that Tax could inhibit the transcriptional activity of these factors through competition for CBP (Colgin and Nyborg, 1998; Riou et al., 2000; Van Orden et al., 1999b). To examine this possibility, we used a Tax mutant in the CBP-binding site, K88A (Harrod et al., 1998). However, K88A was still able to repress the transcriptional activity of C/EBPβ (Fig. 5B). To be sure of the specificity of our tests, we used two other Tax mutants (Smith and Greene, 1990), M9 (Fig. 5B) and M21 (data not shown), as negative controls. Effectively, both mutants were unable to repress C/EBPβ activity. Taken together, our results indicate that C/EBPβ and Tax are not in competition for CBP.

C/EBPβ decreases Tax expression from an infectious HTLV-I molecular clone

The above-cited results suggest that C/EBPβ is able to down-regulate Tax-dependent transcription of HTLV-I genome and, then, may diminish Tax synthesis in the infected cells. To determine the influence of C/EBPβ on Tax production in the context of HTLV-I replication, 293T cells were transfected with the full-length clone of HTLV-I, ACH (Kimata et al., 1994), in the presence of the C/EBPβ-expression vector pcDNA-C/EBPβ. ACH has previously been shown to direct the synthesis of the viral antigens and the Tax protein (Kimata et al., 1994). We then compared the production of Tax in the presence or absence of C/EBPβ. As shown in Fig. 6, C/EBPβ effectively suppressed Tax synthesis. In conclusion, our data show that C/EBPβ inhib-

Fig. 5. The transcription function of C/EBPβ is reduced in the presence of Tax. The transcription function of C/EBPβ is reduced in the presence of Tax. (A) Tax represses the transcriptional activity of C/EBPβ. Transient cotransfection assays were carried out and luciferase values normalized as described in the legend of Fig. 1, with 2 μg of pAPLuc (a minimal alkaline phosphatase promoter) or pE_4APLuc (four tandem CCAAT/enhancer boxes cloned upstream of APLuc), 1 μg of pSG-Tax, and 1 μg of pcDNA-C/EBPβ. Values represent the mean ± SD ($n = 3$). Statistical analysis (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) was performed to demonstrate that the difference obtained in the presence and absence of Tax was significant. (B) Tax represses C/EBPβ activity independently of CBP recruitment. Transient cotransfection assays were performed as described above, with C/EBPβ in the presence of the wild -type Tax or the mutants K88A and M9. The showed data correspond to the ratio between C/EBPβ activity detected in the presence of pE_4APLuc compared to the activity in the presence of APLuc. Values represent the mean ± SD ($n = 3$).

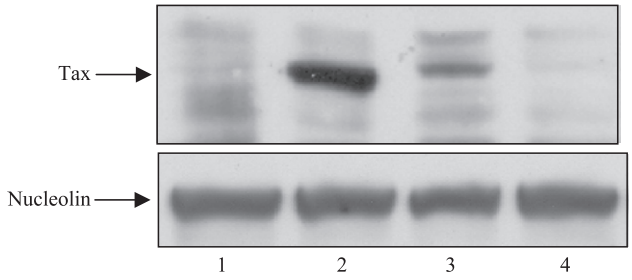


Fig. 6. C/EBPβ inhibits Tax expression from an infectious HTLV-I molecular clone. 293T cells were cotransfected with 2.5 μg of HTLV-I ACH and 0 (lane 2), 2 (lane 3), or 5 μg (lane 4) of pcDNA-C/EBPβ. Lane 1 corresponds to untransfected cells. At 48 h posttransfection, 200 μg of nuclear extracts were electrophoresed through a 10% SDS-PAGE and analyzed by immunoblotting using anti-Tax (top) or anti-nucleolin (bottom).

its Tax expression in vivo from an infectious HTLV-I molecular clone.

Discussion

During the last several years, the key role of the bZIP factors has been clearly established in the Tax-mediated viral transcription (Franklin and Nyborg, 1995; Thébault et al., 2001). As Tax is unable to bind specifically to DNA (Jeang et al., 1988), it interacts with some members of the ATF/CREB family including CREM (Suzuki et al., 1993), CREB (Franklin et al., 1993; Yin and Gaynor, 1996; Zhao and Giam, 1992), and CREB-2 (Gachon et al., 1998; Lemasson et al., 2002; Reddy et al., 1997), and stimulates their DNA-binding activity to the CRE-like motif of the TxREs. In the case of CREB, it has been demonstrated that the amino acid sequence of the basic domain and its ZIP were required for interactions with Tax (Yin et al., 1995). On the other hand, the basic subdomain of the CREB-2 bZIP is not necessary for its association with Tax. Indeed, whereas its ZIP structure is always indispensable, the basic domain of CREB-2 required for the interaction with Tax corresponds to a short basic C-terminal domain located downstream of the ZIP (Gachon et al., 2000). However, it is noteworthy that for both cellular proteins the ZIP structure, which serves as a dimerization interface, is absolutely necessary. Besides, it has been suggested that Tax would stimulate viral transcription by promoting the homodimerization of the ATF/CREB factors (Wagner and Green, 1993).

C/EBPβ, a bZIP factor belonging to the C/EBP family, has also been shown to interact with Tax. Through physical association with two cellular transcription factors, C/EBPβ and Spi-1, Tax *trans*-activates the promoter of human prointerleukin-1β gene (Tsukada et al., 1997). Here, we have analyzed the effect of C/EBPβ on Tax-dependent viral transcription. Unexpectedly, we find that Tax is no more able to *trans*-activate viral transcription is the presence of C/EBPβ and this effect is reciprocal. Different mechanisms can explain this repression. First, the repression could be mediated through competition for binding to the transcriptional cofactor CBP in a mutually exclusive fashion as it has been described for p53 (Van Orden et al., 1999a), p73 (Lemasson and Nyborg, 2001), and MyoD (Riou et al., 2000). Although both proteins interact with CBP, we can exclude this possibility because Tax and C/EBPβ bind to different subdomains of CBP (Harrod et al., 1998; Scoggin et al., 2001). Moreover, the Tax point mutant K88A, which does not interact with CBP, remains capable of repressing C/EBPβ activity. Second, C/EBPβ may form homodimers or heterodimers with CREB-2 (Podust et al., 2001) that may not bind to the TxREs as already suggested for CHOP and CREB-2 (Gachon et al., 2001). However, we cannot apply this model for Tax repression by C/EBPβ because we and others have found that the C/EBPβ-CREB-2 heterodimer form a stable complex on the CRE site (Podust et al., 2001). Third, C/EBPβ may not allow the fixation of Tax on the TxREs. If Tax does not bind to the viral promoter, obviously it cannot stimulate viral transcription. Effectively, taken together, our results show that Tax is no longer able to activate the transcription from the TxREs because the interaction between C/EBPβ and Tax blocks the formation of an active complex on the viral promoter. The incapacity of Tax and C/EBPβ to cooperate together to *trans*-activate viral transcription is probably due to the mode of interaction between the two proteins. As already mentioned in the discussion, Tax stimulates the DNA-binding activity of CREB and CREB-2 by interacting with their ZIP domain. Here, we demonstrate that the bZIP domain of C/EBPβ is not required for the interaction between C/EBPβ and Tax. Moreover, our results seem to be in contrast to the observation that Tax is able to *trans*-activate the promoter of human prointerleukin-1β gene (Tsukada et al., 1997). However, in this case, the effect of Tax is more complex because a mutation in the promoter located at the specific site recognized by Spi-1 abolishes the stimulation by Tax, suggesting that the CCAAT/enhancer motif is not sufficient to allow the activation of the human prointerleukin-1β gene by Tax.

Tax is responsible for a strong cytotoxic-T-lymphocyte response to HTLV-I-infected cells. Therefore, Tax expression has to be tightly regulated, otherwise the infected cells would be eliminated by the host immune system and then unable to survive. Here, we show that C/EBPβ inhibits Tax synthesis in vivo in the context of the full-length provirus replication. Moreover, analysis of C/EBPβ in activated human T cells (Dumais et al., 2002) or primary PBMC (Rosati et al., 2001) revealed an increase in C/EBPβ expression and its rapid accumulation in the nucleus. Taken together, these data suggest that C/EBPβ may help infected T cells to escape the cytotoxic-T-lymphocyte response by inducing a low level of Tax production in infected T cells. Such a model has already been proposed with another cellular bZIP factor, inducible cAMP early repressor (ICER) (Newbound et al., 2000). ICER is a potent transcriptional *trans*-repressor without activation domain that suppresses Tax-dependent tran-

scription (Bodor et al., 1996; Newbound et al., 2000). Indeed, the binding of ICER to TxRE is enhanced by Tax and blocks viral transcription in activated PBMC (Newbound et al., 2000). Moreover, HTLV-I itself codes for two proteins, HBZ and $p30^{II}$, that reduce the viral expression. HBZ down-regulates viral transcription by forming heterodimers with CREB-2 (Gaudray et al., 2002) and c-Jun (Basbous et al., in press) unable to form stable complexes on the viral promoter. $p30^{II}$ acts as a repressor by sequestering CBP (Zhang et al., 2001). In conclusion, all these observations demonstrate that the HTLV-I-infected T cell has developed different strategies to escape the cytotoxic-T-lymphocyte response to maintain a state of persistent infection.

C/EBPβ has also been described to regulate HIV-1 transcription. The HIV-1 LTR has three CCAAT/enhancer sites that bind C/EBPβ (Tesmer et al., 1993). Although it is not necessary for HIV-1 transcription in $CD4^+$ cells (Henderson and Calame, 1997), C/EBPβ is essential for HIV-1 replication in monocytes/macrophages (Henderson et al., 1995). C/EBPβ recruits coactivators to the HIV-1 LTR by physically interacting with histone acetyltransferase complexes and thus participates in remodeling the chromatin organization of the provirus (Lee et al., 2002). If we compare HIV-1 and HTLV-I, C/EBPβ has an opposite effect on the transcriptional regulation of their promoter because it stimulates and represses HIV-1 and HTLV-I transcription, respectively. We have already described the cloning of a cellular factor, the human I-mfa domain-containing (HIC) protein, which has opposite effects on HIV-1 and HTLV-I transcription. Indeed, HIC protein stimulates HTLV-I expression in the presence of Tax but down-regulates expression from HIV-1 LTR in the presence of Tat in CEM cells (Thébault et al., 2000). These differences found between HIV-1 and HTLV-I suggest that these two viruses have developed very different strategies for controlling transcription of their proviral transcription although they utilize common cellular transcription factors.

Materials and methods

Plasmids, transfections, and luciferase assays

The expression vectors pSG-Tax and pBg312HIV-1Lai-Tat, the luciferase reporter plasmids HTLV-I or HIV-1 LTR-luciferase, and pminLUC-viral TxRE have been previously described (Gachon et al., 2001; Thébault et al., 2000). The C/EBPβ-expression vector pcDNA-C/EBPβ and the HTLV-I molecular clone ACH were generous gifts from Kishimoto Tadamitsu and Michael D. Lairmore, respectively. C/EBPβ and the mutant C/EBPβ-bZIP were subcloned into pcDNA3.1/His (Invitrogen). The C/EBPβ truncation mutant without the leucine zipper (C/EBPβ-ΔZIP) was obtained by digesting C/EBPβ cDNA by *Pst*I and the resulted digest was subcloned into pcDNA3.1/His. The description of the luciferase expression plasmids pAPLuc (a minimal alkaline phosphatase promoter) and pE_4APLuc (four tandem C/EBP sites cloned upstream of APLuc) have been published elsewhere (Cooper et al., 1995). CEM cells were transiently cotransfected according to the previously published procedure (Gachon et al., 2000). Five micrograms of pACβ1 was included in each transfection for controlling of the transfection efficiency. The total amount of DNA in each transfection was the same, the balance being made with empty plasmids. Cell extracts equalized for protein content were used for luciferase and β-galactosidase assays. The 293T cells were transfected with ACH by using the FuGENE 6 Tranfection Reagent as described by Roche Molecular Biochemicals protocol.

Protein expression and purification

The bacterial expression vectors pQE containing either CREB-2 or Tax were transformed into *Escherichia coli* M15. The N-terminal 6xHis-tagged proteins were purified as described by the manufacturer (Qiagen). C/EBPβ cDNA was subcloned into pGEX and transformed into *E. coli* BL21 to produce a GST-C/EBPβ fusion protein, which was purified as described by the manufacturer (Amersham Biosciences). The purified proteins were dialyzed against binding buffer without BSA and kept at −80 °C.

Streptavidin–biotin complex assay

The assay was performed as already described (Gachon et al., 2000). Briefly, biotinylated oligonucleotides corresponding to the HTLV-I 21-bp repeat TxRE III (5′-TCGA-CGTCCTCAGGCGTTGACGACAACCCCTCAC-3′) were annealed with their complementary oligonucleotides to form a double-stranded DNA. Biotinylated double-stranded DNA was incubated with bacterially produced proteins in 200 μl of binding buffer containing 50 mM Tris, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 0.1% Triton, 5% glycerol, and 10 mg/ml BSA for 2 h at room temperature before addition of streptavidin beads (Pierce). After 1 h incubation at 4 °C, the beads were extensively washed with binding buffer without BSA. The proteins which remained bound to the beads were eluted in SDS loading buffer and analyzed by Western blotting.

Western blot assay and antisera

Proteins were electrophoresed onto SDS-polyacrylamide gel (SDS-PAGE) and blotted to polyvinylidene difluoride membranes (Millipore). The blot was then incubated for 1 h at room temperature with a blocking solution (Tris-buffered saline containing 5% milk) before addition of antiserum. After 2 h at 20 °C, the blot was washed four times with Tris-buffered saline–0.2% Tween 20 and incubated for 1 h with either goat anti-mouse or goat anti-rabbit immunoglobulin–peroxydase conjugate (Beckman Coulter). After three washes, the membrane was incubated with

enhanced chemiluminescent substrate for detection of peroxydase (Pierce). The membrane was then exposed for 0.5–5 min to hyperfilms-ECL (Amersham Biosciences).

The anti-nucleolin monoclonal antibody and the anti-CREB-2 and anti-C/EBPβ polyclonal sera were purchased from Santa Cruz Biotechnology Inc., the anti-Xpress monoclonal antibody from Invitrogen, and the anti-Tax antibody was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: HTLV-I Tax hybridoma 168A51-42 (Tab176) from Dr. B. Langton.

GST-pull down assay

CREB-2 cDNA cloned into pCI-neo and Tax cDNA cloned into pSG (Gachon et al., 1998) were transcribed and translated in the presence of [^{35}S]methionine and [^{35}S]cysteine using the TNT T7 coupled reticulocyte lysate system of Promega, and incubated at 4 °C with equal amounts of GST-C/EBPβ fusion proteins or GST immobilized on glutathione Sepharose beads (Bulk GST purification Module of Amersham Biosciences) in a buffer containing 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 250 mM NaCl, and 0.1% Nonidet P-40. After 2 h of incubation, the beads were washed five times with incubation buffer and the bound proteins were analyzed by SDS-PAGE followed by autoradiography.

Acknowledgments

This work was supported by institutional grants from the Centre National de la Recherche Scientifique (CNRS) and the Université Montpellier I (UM I), and grants to J.M.M. from the Fondation de France and the Association pour la Recherche sur le Cancer (ARC 5933). G.G. and P.H. are supported by fellowships from the Ministère de l'Education Nationale, de le Recherche et de la Technologie (MENRT) and the Comité Régional (Hérault and Lozère) de la Ligue contre le Cancer, respectively. We thank Kishimoto Tadamitsu, Kathryn Calame, and Michael D. Lairmore for kind gift of the C/EBPβ-expression vector pcDNA-C/EBPβ, the luciferase expression plasmids pAPLuc and pE$_4$APLuc, and the full-length clone of HTLV-I ACH, respectively.

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